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# **Exhibit A** (8 pages)

# Therapeutic Efficacy of OX-40 Receptor Antibody Depends on Tumor Immunogenicity and Anatomic Site of Tumor Growth<sup>1</sup>

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## ABSTRACT

The OX-40 receptor (OX-40R) is a cell surface glycoprotein of the tumor necrosis factor receptor family that is expressed primarily on activated CD4 T cells. Engagement of OX-40R by the OX-40 ligand (OX-40L) is known to costimulate the production of cytokines by activated T lymphocytes and to rescue effector T cells from activation-induced cell death. It was previously reported that *in vivo* ligation of OX-40R by administration of OX-40L:immunoglobulin fusion protein or OX-40R monoclonal antibody (mAb) resulted in a significant prolongation of survival of tumor-bearing mice in four histologically distinct solid tumors. In this study, we demonstrate that the therapeutic efficacy of OX-40R mAb was influenced by the tumor burden, the intrinsic immunogenicity of the tumor as well as by the histological site of tumor growth. Whereas subdermal and intracranial growth of weakly immunogenic MCA 203 and MCA 205 sarcomas and GL261 glioma were susceptible to the mAb treatment, established pulmonary MCA 205 metastases were refractory to the same regimen of treatment. Furthermore, the mAb administration had no impact on the growth of the poorly immunogenic B16/D5 melanoma. Tumor regression mediated by OX-40R mAb was dependent on the participation of both CD4 and CD8 T cells and as a result of tumor rejection, a long-term tumor-specific immunity was established. Analysis of tumor-infiltrating T cells revealed the presence of a far greater number of OX-40R<sup>+</sup> T cells of both CD4 and CD8 phenotypes in the intracranial immunogenic GL261 glioma than that in the poorly immunogenic B16/D5 melanoma. These results suggest that ligation of OX-40R on activated T cells *in situ* in the tumor may provide a necessary costimulatory signal to augment immune responses leading to tumor regression and immunological memory.

## INTRODUCTION

An important goal of cancer immunology and immunotherapy is to understand fundamental principles of immune responses to tumors. This will lead to the design of strategies to enhance tumor antigen recognition by cells of the immune system and to channel reactive pathways for therapeutic benefits. Rejection of tumors through immune responses is primarily mediated by T cells. Growing evidence indicates that T cells require at least two physiologically distinct signals to become activated (1, 2). The first signal is generated by the interaction of T cell antigen receptors and antigenic peptide/MHC class I or II complexes on APCs<sup>3</sup>, e.g., tumor cells or dendritic cells. The second signal is delivered by costimulation molecules on APCs through their counter receptors on T cells (3–6). Without costimulation, exposure of T cells to antigen may induce unresponsiveness or anergy (7, 8). Thus, augmentation of costimulation has been an

attractive approach to enhance weak immune responses during progressive tumor growth.

There are several known molecules that can provide costimulation (5, 9). The best characterized costimulatory signal is the one delivered to the T-cell CD28 receptor by its ligand B7.1 (CD80) or B7.2 (CD86). In many murine tumors, transduction of tumor cells to express B7.1 or B7.2 resulted in the loss of tumorigenicity (10–12). Immunization with such modified tumor cells elicited protective immunity against challenges with wild-type tumors and in some cases, mediated regression of existing tumors (10). In addition, several other membrane-bound receptor-ligand pairs can also serve to be costimulators for T-cell activation. In particular, members of the tumor necrosis factor receptor superfamily have been shown to share the ability to enhance or costimulate the process of T-cell activation (13–17). This family consists of the CD30, CD40, CD27, Fas (CD95), DR3, 4–1BB, and OX-40. The 4–1BB receptor binds to a high-affinity ligand (4–1BBL) expressed on several APCs such as dendritic cells, macrophages, and activated B cells. Expression of 4–1BB is somewhat restricted to primed CD4, CD8 T cells, and natural killer cells (18). Of particular significance is the observation that administration of 4–1BB mAb as a single agent could eradicate well-established tumors in mice (19). Although both CD4 and CD8 participated in the antitumor immune responses, the stimulation of a CD8 CTL response was particularly striking. The CTL activity generated from 4–1BB mAb-treated mice was increased up to approximately 65 times compared with that of spleen cells from control animals. Thus, ligation of costimulation receptors *in vivo* may augment natural immunity to the growing tumors sufficient to induce their regression.

Until recently, the OX-40 receptor-ligand costimulation system has received relatively little attention for exploitation of its ability to enhance antitumor immunity because due to the fact that the receptor expression was reportedly confined to primed CD4 T cells only (20). The OX-40R has a very distinct pattern of expression in animals with EAE. At the inflammatory site, it appears that T cells expressing the OX-40R are cells that recognized the autoantigen and were involved in the pathogenesis of EAE (21–23). Additional analyses of tumor-infiltrating lymphocytes and tumor-draining lymph nodes from melanoma, breast cancer, and head and neck cancer patients identified the presence of OX-40R<sup>+</sup> cells (24, 25). These findings along with our recent demonstration that tumor-specific CD4 T cells isolated from L-selectin<sup>low</sup> cell population of tumor-draining lymph nodes mediated tumor regression without the participation of CD8 T cells (26) have inspired the inception of the hypothesis that *in vivo* ligation of the OX-40R on T cells may augment of antitumor immunity.

Recent work has tested the therapeutic efficacy of the OX-40L: immunoglobulin fusion protein as well as specific OX-40R mAb for the treatment of four antigenically and histologically distinct murine tumors (25). In each tumor, treatment by ligation of OX-40R *in vivo* resulted in a significant improvement in survival of the tumor-bearing mice. The anti-OX-40R effects were dose-dependent and immunologically mediated. In the current study, we further analyzed the immunomodulatory function of the *in vivo* administered OX-40R mAb in mice bearing tumors of various immunogenicities as well as tumors inoculated at different anatomical sites. Our results suggest that the

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<sup>3</sup>The abbreviations used are: APC, antigen-presenting cell; mAb, monoclonal antibody; EAE, experimental autoimmune encephalomyelitis; OX-40R, OX-40 receptor; OX-40L, OX-40 ligand; CM, complete medium; s.d., subdermal/subdermally; BBB, blood-brain barrier; IL, interleukin.

therapeutic responses may be predicated to the extent of OX-40R<sup>+</sup> T lymphocytes infiltrating the growing tumor.

## MATERIALS AND METHODS

**Animals.** Female C57BL/6N (B6) mice, 6–8 weeks old, were purchased from the Biological Testing Branch, Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, MD). They were maintained in a specific pathogen-free environment according to National Institutes of Health guidelines and were used for experiments at the age of 8–12 weeks.

**Tumors.** The MCA 203 and MCA 205 fibrosarcomas are 3-methylcholanthrene-induced tumors of B6 origin (27). The tumors have been routinely passed *in vivo* by serial s.c. transplantation in syngeneic mice and were used within the fifth to the eighth transplantation generation. Single-cell suspensions were prepared from solid tumors by digestion with a mixture of 0.1% collagenase, 0.01% DNase, and 2.5 units/ml hyaluronidase (Sigma, St. Louis, MO) for 3 h at room temperature. The cells were filtered through a layer of no. 100 nylon mesh, washed, and resuspended in HBSS. B16/D5 is a poorly immunogenic subclone of the spontaneously arising B16/BL6 melanoma (28). The B16/D5 tumor does not exhibit a detectable level of MHC class I (H-2 D<sup>b</sup> and K<sup>b</sup>) and class II molecules. These tumor cells were maintained in culture in CM. CM consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1  $\mu$ M sodium pyruvate, 2 mM fresh L-glutamine, 100  $\mu$ g/ml streptomycin, 100 units/ml penicillin, 50  $\mu$ g/ml gentamicin, and 0.5  $\mu$ g/ml fungizone (all from Life Technologies, Grand Island, NY), and  $5 \times 10^{-5}$  M 2-mercaptoethanol (Sigma). GL261 glioma, originally induced by intracranial implantation of methylcholanthrene pellets in a B6 mouse, was obtained from the Division of Cancer Treatment Repository (Frederick, MD; 29). The GL261 tumor cells were maintained in continuous culture in CM. Cultured B16/D5 or GL261 tumor cells were harvested after a short incubation at 37°C with a solution containing 0.25% trypsin and 0.02% EDTA (Life Technologies, Inc., Grand Island, NY). The tumor cells were washed and resuspended in HBSS for animal inoculation.

**Tumor Inoculation.** B6 mice were given s.d. injections with  $1 \times 10^5$  to  $1.5 \times 10^6$  MCA 205 tumor cells suspended in 100  $\mu$ l of HBSS to initiate tumor growth. The diameters of s.d. tumors were measured twice weekly with a Vernier caliper, and size was recorded as an average of perpendicular measurements and presented as individual tumor growth curves. Mice were killed when the dermal tumor reached a size greater than 20 mm. To establish intracranial tumors, B6 mice were anesthetized with 0.8 mg of pentobarbital i.p. and inoculated with 10  $\mu$ l of tumor cell suspension transcranially using a 27-gauge needle and glass tuberculin syringe (Perfectum; Popper & Sons, Inc., New Hyde Park, NY). The needle insertion was perpendicular to the skull and in line with the anterior margin of the ear and the medial half of the right eye. The depth of insertion was controlled by placement of electric wire insulation as a collar over the needle with exposure of the terminal 4 mm. To establish pulmonary metastases, mice were given i.v. injections with  $1 \times 10^5$  MCA 205 tumor cells suspended in 1 ml of HBSS. On day 21, mice were killed, and metastatic tumor nodules on the surface of the lung were enumerated after counterstaining with India ink.

**mAb to OX-40R and Treatment Protocol.** Hybridoma that produced the antimurine OX-40R-specific mAb (termed OX-86) was obtained from the European Collection of Animal Cell Cultures (30). The hybridoma was grown in RPMI medium containing 10% FCS that was depleted of immunoglobulin by passing it over protein G columns. The cells were grown to high density, and the supernatant was poured over a protein G column, and purified antibody was eluted. Antibody concentrations were quantitated by absorption at 280 nm. In most experiments, tumor-bearing mice were treated with the mAb on days 3 and 7 after tumor inoculation by i.p. injections. Doses of mAb are indicated in the figure legends.

**Depletion of T-Cell Subsets *in Vivo*.** Ascites for the rat IgG2b mAb against murine CD4 (GK1.5, L3T4) and CD8 (2.43, Lyt 2.2) was produced in sublethally irradiated (500 R), cyclophosphamide (2 mg/mouse)-treated DBA/2 mice. Mice with 3-day established intracranial GL261 tumors were depleted of T-cell subsets by i.v. injection of 0.2 ml of ascites fluid diluted to 1.0 ml with HBSS prior to OX-40R mAb treatment. T cell depletion was confirmed by flow cytometric analyses of spleen cells from treated animals.

**Isolation and Characterization of T Cells from Intracranial Tumors and Lymphoid Organs.** Anesthetized B6 mice with 10-day established GL261 or B16/D5 intracranial tumors were perfused through the left ventricle of the heart with 3 ml of HBSS before removal of the brain. Single-cell suspensions were prepared by digesting minced brain tissue in 40 ml of HBSS containing 10 mg collagenase (type IV, Sigma) for 60 min at room temperature. The cell suspensions were washed in HBSS, resuspended in 10 ml of 50% Percoll (Pharmacia, Uppsala, Sweden), and then overlaid with 4 ml of 30% Percoll to form a discontinuous gradient in a 15-ml centrifuge tube. The gradient was centrifuged at 4°C for 40 min at  $1000 \times g$ . Cells recovered from the interphase were washed in HBSS before flow cytometric analysis. Single-cell suspensions from lymph nodes or spleens were prepared mechanically by teasing organs with needles followed by pressing tissue fragments with the blunt end of a plastic syringe. Cells were stained by indirect immunofluorescence for the expression of OX-40R using OX40L:immunoglobulin fusion protein and FITC-conjugated antihuman IgG (Caltag, South San Francisco, CA). Cells were also stained with PE-conjugated anti-CD4 or CD8. The membrane fluorescence was analyzed using FACSCalibur (Becton Dickinson, Sunnyvale, CA).

**Statistical Analysis.** The significance of differences in numbers of pulmonary metastases between groups was analyzed by the Wilcoxon rank-sum test. Differences of numbers of cells infiltrating tumor tissues were analyzed by the Student's *t* test. A two-tailed *P* of  $\leq 0.05$  was considered significant.

## RESULTS

**Tumor Rejection Induced by OX-40R Antibody Administration.** In a recent report, the therapeutic effects of *in vivo* administration of either OX-40L:immunoglobulin fusion protein or OX-40R-specific mAb in four histologically distinct murine tumors have been demonstrated (25). The effects appear to be affected by the intrinsic immunogenicity of the experimental tumor. In the present study, we further extended these observations by examining the therapeutic effects of OX-40R mAb in different animal models and in situations in which tumors were growing at different anatomical sites. We initially treated mice bearing s.d. tumors of the weakly immunogenic MCA 205 fibrosarcoma with OX-40R mAb (150  $\mu$ g i.p.) on days 3 and 7. Fig. 1 depicts the results in which mice inoculated with  $1 \times 10^5$  (Fig. 1, *Expt. 1*) or  $3 \times 10^5$  (Fig. 1, *Expt. 2*) MCA 205 tumor cells responded to the treatment resulting in complete tumor eradication in 3 of 5 animals. In mice inoculated with  $1.5 \times 10^6$  tumor cells (Fig. 1, *Expt. 3*), treatment with OX-40R mAb resulted in some retardation and delay of tumor growth, but all eventually succumbed to the progressive tumors. In all of the tumor doses, inoculation led to progressive s.d. tumor growth in control mice. These results, thus, indicate that tumor burden may be a limiting factor to the therapeutic response of the OX-40R mAb treatment.

**Therapeutic Efficacy of OX-40R mAb for the Treatment of Intracranial Tumors.** Although the brain has long been considered to be an immunologically privileged site, our extensive studies have demonstrated the successful treatment of experimental intracranial tumors by the systemic transfer of activated tumor-specific immune T cells (26, 31). We, therefore, examined the therapeutic effects of OX-40R mAb against intracranial tumors. Of the three weakly immunogenic tumors, MCA 205, MCA 203 sarcomas, and GL261 glioma, intracerebral inoculations of  $1 \times 10^5$  tumor cells consistently resulted in progressive growth of tumors in the brain and killed the host with a median survival time of approximately 20 days. Mice bearing MCA 205 intracranial tumors responded to the treatment with OX-40R mAb and demonstrated the efficacy of therapy was antibody dose-dependent. Treatment with 150  $\mu$ g mAb i.p. on days 3, 7, and 11 prolonged the survival, but only one of six treated animals was cured (Fig. 2A). Treatment with higher antibody doses, either by four administrations of 150  $\mu$ g on days 3, 7, 11, and 15 or by two administrations of 300  $\mu$ g on days 3 and 7, demonstrated a higher

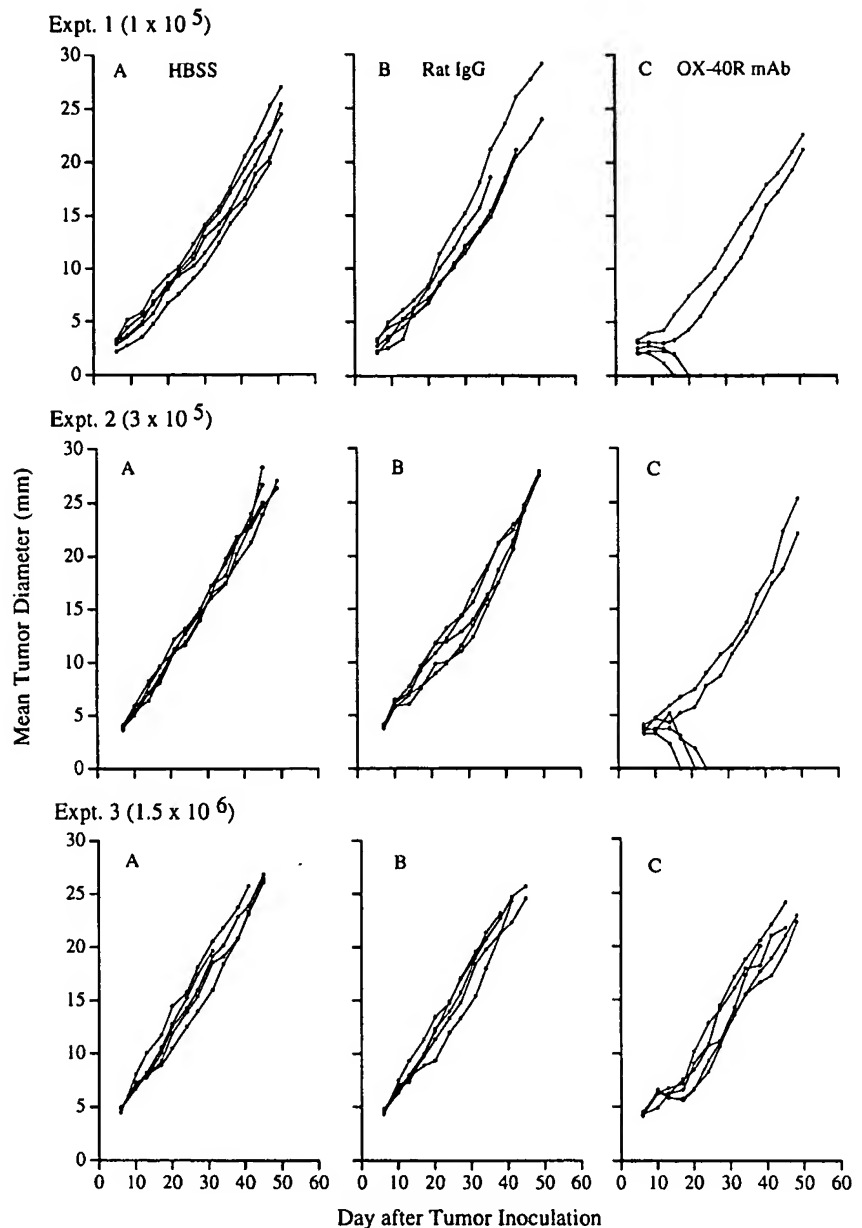


Fig. 1. Induction of antitumor immunity by OX-40R mAb against s.d. tumors. B6 mice in groups of five were inoculated s.d. with  $1 \times 10^5$  (Expt. 1),  $3 \times 10^5$  (Expt. 2), or  $1.5 \times 10^6$  (Expt. 3) MCA 205 tumor cells. On days 3 and 7, mice were treated by i.p. injections of HBSS (A), control rat IgG (150  $\mu$ g/injection; B), or OX-40R mAb (150  $\mu$ g/injection; C). Tumor sizes were estimated by measuring perpendicular diameters, and the results are expressed as mean diameters of tumors in individual mice.

therapeutic efficacy resulting in complete eradication of tumors in 50% of the treated mice. Similarly, for the treatment of MCA 203 sarcoma and GL261 glioma, i.p. injection of mice with 300  $\mu$ g of OX-40R mAb on days 3 and 7 prolonged survival, and 3 and 4 of 6 treated animals were cured of tumors, respectively (Fig. 2, B and C). Increasing the dose of GL261 tumor cells in the tumor inoculum resulted in decreased therapeutic effects of the antibody treatment (Fig. 2D). In an additional experiment, mice inoculated intracerebrally with  $10^5$  MCA 205 tumor cells were treated with two injections of 300  $\mu$ g of OX-40R mAb 4 days apart commencing on day 3, 5, or 7. Whereas 2 of 5 mice treated beginning on day 3 were cured, all of the mice succumbed to the progressive growing tumors when treatment was delayed (data not shown). These results thus confirm that the tumor burden is a limiting factor as seen in previously experiments (Fig. 1).

In an attempt to establish a model system for the treatment of poorly immunogenic tumors, we used a cloned tumor cell line, B16/D5, which was derived from the B16/F10/BL6 melanoma. Previous experimental results suggested that the B16/D5 tumor failed to im-

mune syngeneic animals using irradiated tumor cells or admixed with *Corynebacterium parvum* as an adjuvant. In addition, lymph nodes draining the B16/D5 tumor contained very few immune effector cells when adoptively transferred to treat 3-day established pulmonary metastases (32). In the present study, intracerebral inoculation of as few as  $1 \times 10^3$  tumor cells led to progressive tumor growth that eventually killed the animals, with a median survival time of 18 days. Treatment of intracranial B16/D5 tumor-bearing mice with two i.p. administration of 300  $\mu$ g OX-40R mAb on days 3 and 7 did not demonstrate any therapeutic effects (Fig. 2E).

A similar strategy was used to treat pulmonary metastases derived from the MCA 205 sarcoma. Mice were injected i.v. with  $1 \times 10^5$  tumor cells suspended in 1.0 ml of HBSS to establish multiple metastases in the lung. Such a model system has been used extensively in the past for evaluating therapeutic efficacy of both active and adoptive immunotherapies. Mice bearing pulmonary metastases were treated exactly as described above with two i.p. injections of 300  $\mu$ g of OX-40R mAb on days 3 and 7. On day 21, all of the mice were killed and metastatic nodules on the surface of the lung were esti-

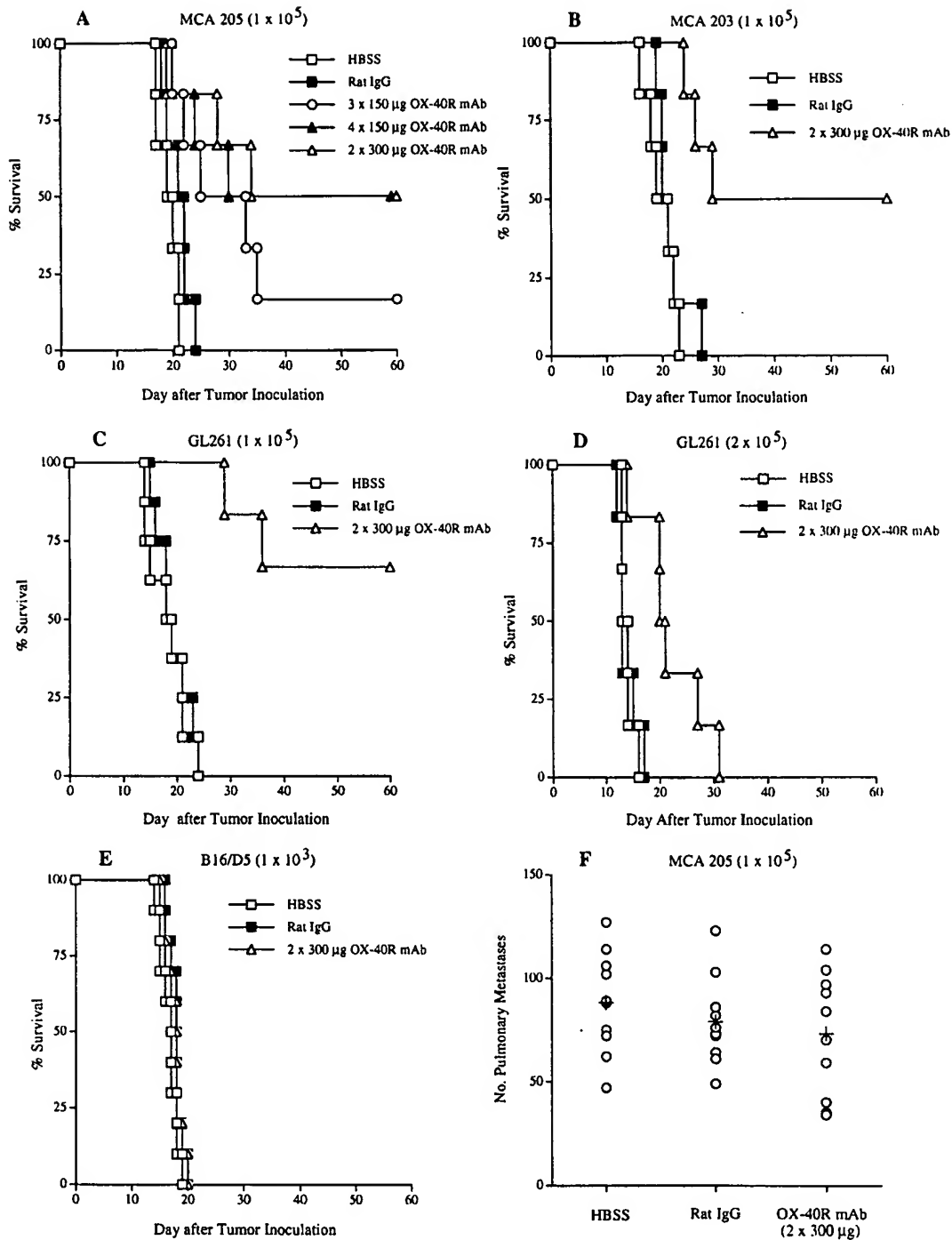


Fig. 2. Antitumor effects of OX-40R mAb administration against intracranial tumors. In A, B6 mice in groups of six were inoculated intracranially (i.e.) with  $1 \times 10^5$  MCA 205 tumor cells. They were treated by multiple i.p. injections of HBSS on days 3, 7, 11, and 15 (□), rat IgG (■, 300  $\mu$ g/injection on days 3 and 7), or OX-40R mAb (○, 150  $\mu$ g/injection on days 3, 7, and 11; ▲, 150  $\mu$ g/injection on days 3, 7, 11, and 15; △, 300  $\mu$ g/injection on days 3 and 7). In B, B6 mice in groups of six were inoculated i.c. with  $1 \times 10^5$  MCA 203 tumor cells. On days 3 and 7, mice were treated by i.p. injections of HBSS, rat IgG (300  $\mu$ g/injection), or OX-40R mAb (300  $\mu$ g/injection). In C and D, B6 mice in groups of six were inoculated i.c. with  $1 \times 10^5$  (C) or  $2 \times 10^5$  (D) GL261 glioma cells. On days 3 and 7, mice received i.p. injections of HBSS, rat IgG (300  $\mu$ g/injection), or OX-40R mAb (300  $\mu$ g/injection). In E, B6 mice in groups of 10 were inoculated i.c. with  $1 \times 10^3$  B16/D5 melanoma cells. On days 3 and 7, mice were treated by i.p. injections of HBSS, rat IgG (300  $\mu$ g/injection), or OX-40R mAb (300  $\mu$ g/injection). In F, B6 mice in groups of five were inoculated i.v. with  $1 \times 10^5$  MCA 205 tumor cells to establish pulmonary metastases. On days 3 and 7, mice were treated by i.p. injections of HBSS, rat IgG (300  $\mu$ g/injection), or OX-40R mAb (300  $\mu$ g/injection). Compilations of two independent experiments because of similar results.

ated. Despite its effectiveness for the treatment of s.d. and intracranial MCA 205 tumors, the antibody failed to effect tumor growth as judged by the numbers of metastatic nodules in treated mice as compared with controls (Fig. 2F).

Cumulatively, this series of experiments confirms the therapeutic effectiveness of OX-40R ligation in the induction of tumor eradication as claimed previously (25). The successful treatment is mAb dose-

dependent and effected by the intrinsic immunogenicity of tumors. It is also evident that the response of a particular tumor to the treatment varies and is dependent on the histological location of tumor growth.

**Specificity and Long-Term Immunity after Successful Treatment.** To determine whether a long-lasting immunity was induced in mice that were cured by the OX-40R mAb treatment, we challenged survival animals, 60 days after initial tumor inoculation, with either

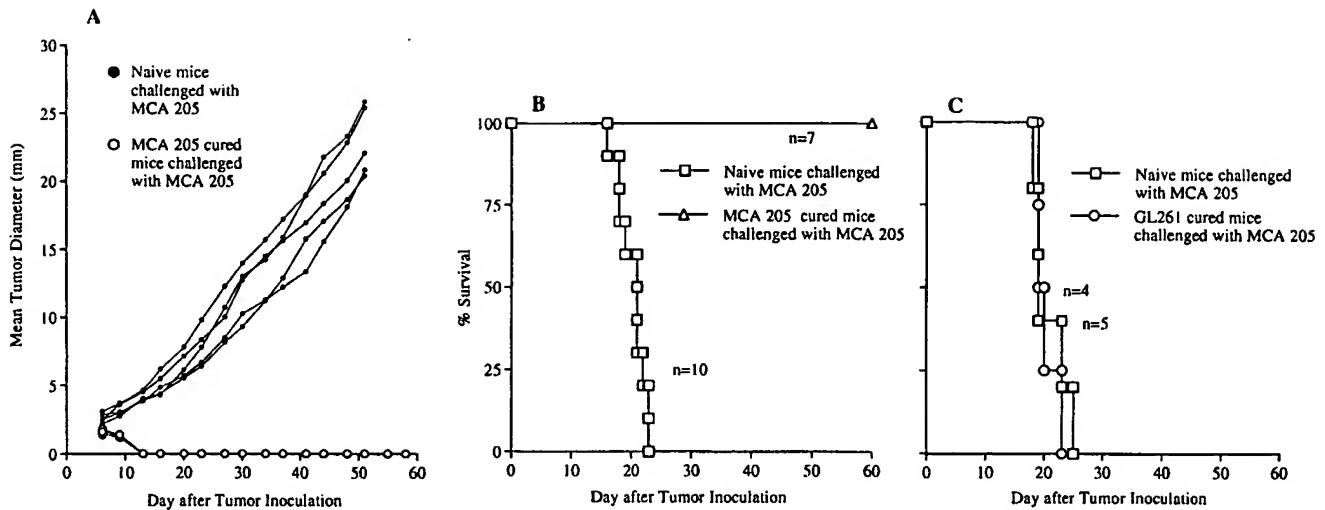


Fig. 3. Induction of long-term protective immunity. In A, four B6 mice that had been cured of the s.d. MCA 205 tumor, and remained tumor-free for 60 days, were rechallenged s.d. with  $3 \times 10^5$  MCA 205 tumor cells (O). After a period of 8 days transient growth, all of the tumor nodules regressed, as compared with progressive tumor growth in all of the control normal mice (●). In B, the seven mice from the Fig. 2A experiment that had been cured of intracranial (i.c.) MCA 205 tumor, and remained tumor-free for 60 days, were rechallenged i.c. with  $1 \times 10^5$  MCA 205 ( $\Delta$ ). Naive normal mice were similarly challenged ( $\square$ ). In C, the four mice from the Fig. 2C experiment that had rejected the i.c. GL261 tumor, and remained tumor-free for 60 days, were challenged i.c. with  $1 \times 10^5$  MCA 205 (O). Naive normal mice served as control ( $\square$ ).

the same tumor or an antigenically distinct tumor. All of the mice cured of MCA 205 primary s.d. tumors (see Fig. 1) resisted a s.d. rechallenge with  $3 \times 10^5$  MCA 205 tumor cells, whereas control, naive mice showed progressive tumor growth after tumor challenge (Fig. 3A). Similarly, mice that survived the intracranial MCA 205 tumors were resistant to a second intracranial challenge ( $1 \times 10^5$ ) with the same tumor (Fig. 3B). We also tested the specificity of this long-term immunity by rechallenging the mice that survived the intracranial GL261 tumor as a consequence of OX-40R mAb treatment with the antigenically distinct MCA 205 tumor. As can be seen in Fig. 3C, the growth of MCA 205 intracranial tumors has not been affected by the initial rejection of the GL261 tumor. These results indicate that the tumor eradication triggered by OX-40R mAb established a long-lasting tumor-specific immunological memory.

**Role of T-Cell Subsets in OX-40R mAb-induced Antitumor Response.** The above experiments strongly suggest that tumor eradication by OX-40R mAb treatment was an indirect result of the augmentation of antitumor immune responses. Because specificity and long-term immunity are maintained by T cells, we examined the role of different T-cell subsets in OX-40R mAb-mediated tumor regression. Mice inoculated intracranially with GL261 tumor cells were treated with two courses of OX-40R mAb (300  $\mu$ g/each) i.p. on days 3 and 7. Cohorts of treated mice were also injected i.v. with CD4 (GK1.5) or CD8 (2.43) mAbs to deplete corresponding T-cell subsets on day 3 prior to the first OX-40R mAb treatment. The method of *in vivo* depletion of T-cell subsets with mAb has been a routine procedure in our laboratory. Flow cytometric analysis of spleen cells 14 days after mAb T-cell depletion revealed a reduction from  $\sim 20\%$  in untreated mice to  $< 2\%$  of CD4 or CD8 T cells in the treated mice. In the experiment depicted in Fig. 4, mice treated with OX-40R mAb without T-cell depletion showed prolongation of survival and three of six mice were cured of the tumor. In animals depleted of either CD4 or CD8 T cells, the antitumor effects of the OX-40R mAb were abrogated. Although OX-40R has been primarily expressed on activated CD4 T cells, and the GL261 glioma does not express MHC class II molecules, our results suggest that tumor regression induced by the OX-40R mAb treatment required the participation of both CD4 and CD8 host T cells.

**Expression of OX-40R on Tumor-infiltrating T Lymphocytes and the Susceptibility of the Tumor to mAb Treatment.** Successful treatment of tumors by the OX-40R ligation with mAb seemed to be influenced by the intrinsic immunogenicity of the target tumor (see Fig. 2). One possible mechanism responsible for the tumor susceptibility is the abundance of tumor-sensitized precursor T cells during the growth of immunogenic tumors. We, therefore, attempted to define the OX-40R expression on tumor-associated T cells isolated from 10-day-old tumors of both the susceptible immunogenic GL261 glioma and the resistant poorly immunogenic B16/D5 melanoma. Because successful OX-40R mAb treatment resulted in tumor-specific systemic immunity, we also examined the expression of OX-40R on splenic T cells from tumor-bearing mice. Quantitatively, in the intracranial GL261 tumor, there were  $\sim 25\%$  tumor-associated T cells, which is 6-fold more T cells than could be detected in the B16/D5 tumor ( $\sim 4.5\%$ , Table 1). It is noteworthy that among tumor-infiltrating T cells, there were  $26 \pm 3\%$  of the GL261 tumor-associated CD4

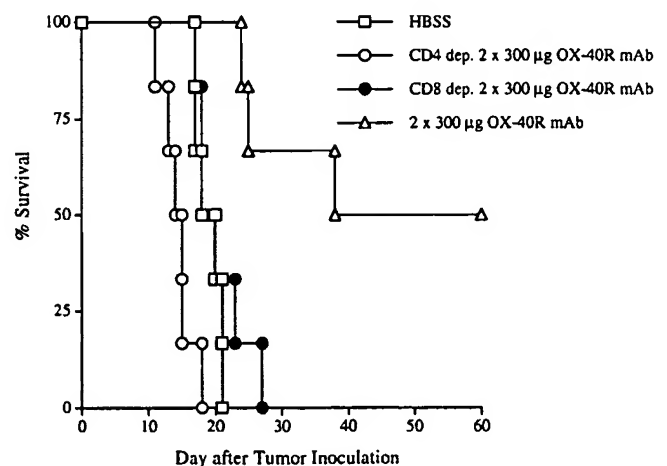


Fig. 4. Role of CD4 and CD8 T cells in OX-40R mAb induced intracranial (i.c.) tumor regression. B6 mice with 3-day GL261 i.c. glioma were depleted of CD4 or CD8 T cells by i.v. injections of GK1.5 (O) or 2.43 (●) mAb, respectively, on day 3, 2 h before i.p. treatment with OX-40R mAb (300  $\mu$ g/injection). The mice were treated again by i.p. administration of OX-40R mAb on day 7 (300  $\mu$ g/injection).

Table 1 Contents of OX-40R<sup>+</sup> T cells in tumor mass<sup>a</sup>

Tumor	% T cells in tumor mass		% OX-40R expression on	
	CD4	CD8	CD4	CD8
GL261	13.6 ± 2.5	11.1 ± 1.3	26.3 ± 3.4	10.7 ± 3.0
B16/D5	2.8 ± 1.0	1.7 ± 1.0	11.3 ± 3.3	3.9 ± 1.0

<sup>a</sup> Average of three independent experiments.

cells expressing OX-40R, whereas only  $11 \pm 3\%$  of OX-40R<sup>+</sup> CD4 cells were detected in the B16/D5 tumor (Fig. 5). Unexpectedly,  $11 \pm 3\%$  and  $4 \pm 1\%$  of CD8 cells isolated from GL261 and B16/D5 tumors were OX-40R<sup>+</sup>, respectively. Analysis of OX-40R expression on splenic T cells of GL261-bearing mice revealed that  $\sim 15\%$  of CD4 cells expressed OX-40R, whereas only  $\sim 7\%$  of CD4 T cells from B16/D5-bearing mice were OX-40R<sup>+</sup>. In both tumor systems, no OX-40R<sup>+</sup> CD8 T cells were detected in the spleen (Fig. 5) or lymph nodes (data not shown). It is possible that OX-40R<sup>+</sup> T cells in the tumors are the candidate for targeting by the mAb. The observations that the therapeutic efficacy is dependent on both the CD4 and CD8 T cells and the immunogenicity of the treated tumor, support this possibility.

## DISCUSSION

The OX-40R has been reported to be expressed on T cells isolated from the inflammatory site in several inflammatory diseases, including rheumatoid arthritis, graft-versus-host disease, and EAE, which suggests that signaling through OX-40R may be involved in modulating immune reactions (21–23, 33). In murine models, blockade of the OX-40R signaling has been described to

ameliorate the pathogenesis of EAE (34) and hapten-induced colitis (35), and, more recently, to attenuate alloantigen-specific CTL response (36). These observations underscore the role of OX-40R signaling in the regulation of immune responses. However, very little information is available with regard to its role in the immune response to malignancies. This may largely reflect the restriction in the expression of OX-40R, which seemed to confine to CD4 T lymphocytes (20), and the well-documented, dominant role of CD8 CTL in the immune response to tumors. It was not until recently that CD4 T cells alone were demonstrated to mediate the regression of established tumors (26).

The results of current studies have extended previous findings that *in vivo* engagement of OX-40R by administration of OX-40L:immunoglobulin fusion proteins or OX-40R mAb resulted in significant therapeutic benefits in four histologically and immunologically distinct murine tumors (25). Whereas our results emphasized the critical role of the immunogenicity of tumors to the effects of OX-40R mAb treatment, the histological location of tumor growth was also a determining factor affecting the outcome of treatment. It is clear that s.d. and intracranial tumors were susceptible to the antibody treatment in a dose-dependent manner. Established pulmonary metastases from the same tumor seemed to be refractory to the effects of mAb. To explain the difficulty in treating metastases in the lung, it is possible that micrometastases may not facilitate sufficient intratumoral infiltration of activated T cells for mAb targeting as compared with solitary s.d. and intracranial tumors. This is supported by our recent observation, in which therapy with the adoptive transfer of tumor-specific T cells in conjunction with OX-40R mAb administration significantly accelerated the antitumor immunity against 10-day established pulmonary

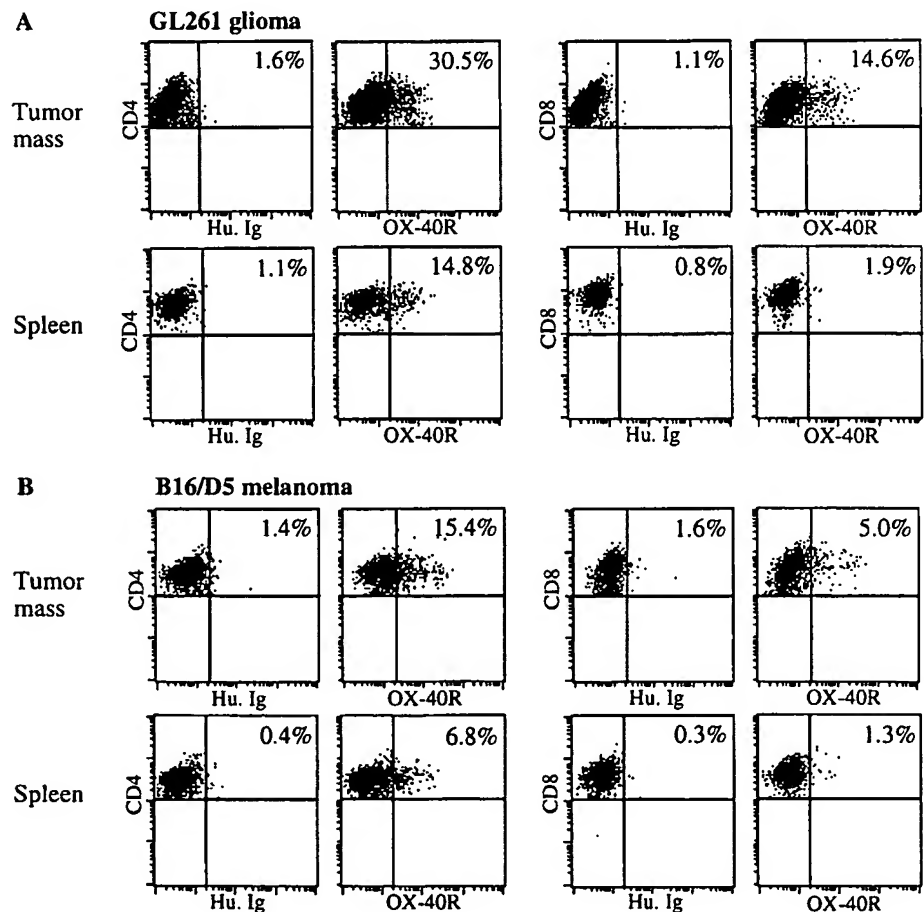


Fig. 5. Expression of OX-40R on T lymphocytes isolated from intracranial GL261 glioma or B16/D5 melanoma. Mononuclear cell suspensions were isolated and stained with murine OX-40L:Fc-immunoglobulin chimeric protein followed with FITC-conjugated anti-human IgG, before being double-stained with either PE-conjugated anti-CD4 or -CD8. By two-color flow cytometry, samples were gated on CD4 or CD8 T cells to display the expression of OX-40R on each subset. Upper right quadrants, the proportions (%) of the CD4 or CD8 T cells expressing OX-40R are indicated. Data are representative of three experiments.



metastases.<sup>4</sup> It is, therefore, possible that advanced pulmonary metastases might be more susceptible to the treatment effects of mAb. This hypothesis is being tested experimentally.

The responsiveness of intracranial tumors to the systemic OX-40R mAb treatment is intriguing because the brain is considered to be an immunologically privileged site because of the existence of the BBB. It is also however, possible that the growing tumor in the brain disrupts the integrity of the BBB, which allows the entry of antibodies. Because the OX-40R mAb does not directly interact with tumor cells, and its function is targeting the activated T cells by ligation of OX-40R on their surface (23), an initial immune response must have occurred to generate activated T cells that express the OX-40R. Although inflammatory cells are rare in the normal brain, increased leukocyte entry occurs in pathological conditions. The physical BBB using tight endothelial junctions and glial end-feet, blocks passive entry of large molecules but does not stop the active entry of living cells (37, 38). Furthermore, in the normal brain, MHC expression is minimal. However, both MHC class I and II expression can be up-regulated on microglia and endothelial cells under the influence of IFN- $\gamma$  and other cytokines (39, 40). These cells may serve the function of APCs; thus, the growing brain tumors may have created a microenvironment conducive to initiating an immune response. Indeed, not only have we observed the expression of OX-40R on the tumor-associated or tumor-infiltrating lymphocytes but the total number of cells and percentages of OX-40R positive cells seems to predict their responses to the OX-40R mAb treatment.

We are aware that the intracerebral route of injection may have damaged the BBB, perhaps permitting local entry of lymphocytes and the mAb. However, this is unlikely to occur solely after a local injury, because there is a lack of inflammatory response in the brain after intracerebral injection of sterile PBS (41). The progressive growth of a tumor, on the other hand, may disrupt the integrity of the BBB even in naturally occurring tumors. It has been reported that the primary human gliomas are often infiltrated with lymphocytes, and in the peripheral circulation, there exists lymphocytes specifically reactive to glioma cells (42, 43). In fact, survival of the glioma patients correlates with the degree of lymphocytic infiltration in the tumor. Nevertheless, we cannot entirely exclude minor injury as a contributing factor in the success of antibody-mediated tumor regression.

The finding that the poorly immunogenic B16/D5 melanoma was not responsive to the therapeutic effects of OX-40R mAb has several explanations. It is possible that B16/D5 tumor cells either lack molecules that can serve sufficiently as tumor antigens recognized by T cells or are deficient in the processing, transportation or presentation of such molecules by APCs. It is, relevant therefore, to note that MHC molecules (both class I and II) are not detectable on B16/D5 cells. In many poorly immunogenic tumors, increasing MHC class I expression by transfection with MHC class I genes or IFN- $\gamma$  cDNA resulted in an enhanced sensitivity to CTL lysis *in vitro* and an increased infiltration of tumor by CD8 lymphocytes *in vivo* (10, 44). Furthermore, B16 melanoma cells transduced with a gene encoding granulocyte-macrophage colony-stimulating factor could elicit a protective immunity against challenges with wild-type tumor cells (45). This finding may be explained by the production of cytokines and/or enhanced expression of MHC and costimulatory molecules induced by granulocyte-macrophage colony-stimulating factor. Taken together, the failure to treat the B16/D5 melanoma may be primarily attributable to insufficient triggering of the initial immune response. If this is the case, transduction of tumor cells to express MHC molecules

or IFN- $\gamma$  may provide a means of increasing responsiveness to the OX-40R mAb treatment against poorly immunogenic tumors.

As discussed earlier, the administration of 4-1BB mAb eradicates established tumors in mice (19). Analysis of mechanisms of tumor rejection revealed the participation of both CD4 and CD8 in tumor-bearing mice. Because the 4-1BB glycoprotein is expressed on both of the activated CD4 and CD8 T cells (18), it is logical to hypothesize that the antibody amplifies both CD4 and CD8 immune responses. In contrast to the 4-1BB, the OX-40R has been repeatedly demonstrated to be preferentially expressed by activated CD4 T cells (20, 23, 24). Our recent studies have demonstrated that appropriately sensitized and activated CD4 T cells alone could mediate potent antitumor responses when adoptively transferred to tumor-bearing mice (26). Because of these findings, we hypothesized a restricted role of OX-40R mAb to be amplifying CD4 T cell-mediated antitumor reactivity. However, T cell phenotype analysis indicated that both CD4 and CD8 T cells were required for effective treatment with the OX-40R mAb. It was first interpreted that OX-40R signaling on CD4 T cells provided an increased T-helper function, which in turn facilitated a CD8 CTL response. To support this hypothesis is the observation that the adoptive transfer of CD8-depleted spleen cells from OX-40L:immunoglobulin cured mice conferred resistance to tumor challenge in naive mice (25). Because of the demonstration of OX-40R<sup>+</sup> CD8 T cells in tumor-infiltrating lymphocytes, it is also possible that the mAb directly binds to CD8 T cells and activates them. CD8 T cells have the potential to express the OX-40R after stimulation with potent mitogens such as ConA and PHA (46).

The mechanism by which administered OX-40R mAb may assist the generation of antitumor responses or enhance existing antitumor immunity is poorly understood. It has previously been shown that sensitized CD4 T cells exposed to Ag and cocultured with B7.1/OX-40L expressing fibroblasts, demonstrate enhanced proliferation, IL-2 secretion, and prolonged survival (47). Thus, one possibility is that the tumor-infiltrating T lymphocytes, sensitized *in vivo* to tumor antigens, were at a stage of differentiation that permitted proliferation and activation by the OX-40R mAb ligation. Because most antigen-sensitized T cells become susceptible to activation-induced cell death, and only a minority differentiate to become memory T cells (48), ligation of the OX-40R on antigen-activated T cells may abrogate or delay the impact of activation-induced cell death leading to increased Ag-specific memory (49). In addition, OX-40R mAb treatment may shift the balance between Th1 and Th2 immune responses. Blocking of OX-40R signaling has resulted in reducing *in vivo* transcript for Th1 cytokines such as tumor necrosis factor- $\alpha$ , IFN- $\gamma$ , IL-2 and IL-12 in animals with EAE and bowel disease (34, 35). However, in studies with naive CD4 T cells, *in vitro* activation by the OX-40L resulted in promoting Th2 cell development (50, 51). Shifting of type 1/type 2 T cell functions as a possible mechanism of OX-40R mAb-mediated tumor regression remains to be determined.

In summary, ligation of OX-40R *in vivo* with antibodies can lead to delaying of tumor progression and, in some cases, eradicating tumors. The demonstration of OX-40R<sup>+</sup> T cells in tumor-infiltrating lymphocytes suggests that they are the targets of antibody binding and activation. Therapeutic efficacy of the antibody, however, seemed to be limited by the tumor burden. Recently, it has been demonstrated that immune responses are regulated, at least in part, by naturally occurring CD4<sup>+</sup>, CD25<sup>+</sup> T cells in normal animal (52). In some animal tumor model systems, depletion of this population of regulatory cells resulted in enhanced immune responses and tumor eradication (53, 54). Because the therapeutic effect of the OX-40R mAb is indirect through the induction of antitumor immune responses, it may

<sup>4</sup> Unpublished observation.



be possible to improve the therapeutic efficacy by the removal of CD4<sup>+</sup>, CD25<sup>+</sup> T cells. This hypothesis is being tested in our laboratory. Finally, the significance of this approach is underscored by the fact that similar OX-40R<sup>+</sup> T cells have been demonstrated in several human malignancies including melanoma, head and neck carcinoma, and carcinoma of the breast (24, 25). If these cells prove to be tumor-sensitized T lymphocytes, ligation of OX-40R mAb may provide tumor Ag-specific therapeutic benefits in cancer patients.

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